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Real-Time PCR Approach for Detection of Environmental Sources of *Campylobacter* Strains Colonizing Broiler Flocks[▽]

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Reducing colonization of poultry flocks by *Campylobacter* spp. is a key strategy in the control and prevention human campylobacteriosis. Horizontal transmission of campylobacters, from in and around the farm, is the presumed route of flock colonization. However, the identification and prioritization of sources are confounded by the ubiquitous nature of these organisms in the environment, their poor rates of recovery by standard culture methods, and the need for cost-effective and timely methods for strain-specific comparison. A real-time PCR screening test for the strain-specific detection of campylobacters in environmental samples has been developed to address this issue. To enable this approach, fluorescently labeled PCR oligonucleotide probes suitable for a LightCycler-based assay were designed to match a highly variable DNA segment within the *flaA* short variable region (SVR) of *Campylobacter jejuni* or *C. coli*. The capacity of such probes to provide strain-specific tools was investigated by using bacterial cultures and spiked and naturally contaminated poultry fecal and environmental samples. The sensitivity of two representative probes was estimated, by using two different *C. jejuni* strains, to be 1.3×10^2 to 3.7×10^2 CFU/ml in bacterial cultures and 6.6×10^2 CFU/ml in spiked fecal samples. The specificity of the SVR for *C. jejuni* and *C. coli* was confirmed by using a panel of strains comprising other *Campylobacter* species and naturally contaminated samples. The approach was field tested by sampling the environment and feces of chickens of two adjacently located poultry houses on a conventional broiler farm throughout the life of one flock. All environmental samples were enriched for 2 days, and then DNA was prepared and stored. Where feasible, campylobacter isolates were also recovered and stored for subsequent testing. A strain-specific probe based on the SVR of the strain isolated from the first positive chicken fecal sample was developed. This probe was then used to screen the stored environmental samples by real-time PCR. Pulsed-field gel electrophoresis was used to compare recovered environmental and fecal isolates to assess the specificity of the method. The results established the proof of principle that strain-specific probes, based on the SVR of *flaA*, can identify a flock-colonizing strain in DNA preparations from enriched environmental cultures. Such a novel strategy provides the opportunity to investigate the epidemiology of campylobacters in poultry flocks and allows targeted biosecurity interventions to be developed. The strategy may also have wider applications for the tracking of specific campylobacter strains in heavily contaminated environments.

Campylobacteriosis is widely considered the major cause of acute bacterial intestinal infectious disease worldwide. *Campylobacter jejuni* and *C. coli* are the major pathogenic species and are ubiquitous in the environment and colonize the gastrointestinal tracts of most wild and domestic animals but appear to have evolved to preferentially colonize the avian gut. Although the sources of human infection remain unclear, the handling of contaminated poultry meat and consumption of undercooked poultry are assumed to be the primary causes of human campylobacteriosis. Reducing colonization in poultry flocks is a key strategy in the control and prevention of human campylobacteriosis (26). Chicks are considered to be hatched free of campylobacters. Colonization of the flock usually occurs at 2 to 3 weeks of age, and several molecular epidemiological studies (3, 21, 33) suggest that colonization is restricted in intensively reared birds to one or two predominant strains, reflecting limited exposure. The route of infection is clearly horizontal from the poultry farm environment. Multiple sources, including wild birds, rodents, farm staff, etc., have been suggested from previous studies, but the relative importance of such sources is unclear. For other organisms, such as *Salmonella*, strategies to identify sources are dependent on culture from the environment and molecular typing to compare environmental and poultry isolates. For poultry campylobacters, this strategy has been unsuccessful, largely because of the ubiquitous nature of the organism in the environment, the weakly clonal nature of the bacterial population structure, and the poor rate of *Campylobacter* recovery from environmental sources.

Over recent years, molecular typing methods have been applied to attempt the identification of potential sources of flock infections (12, 13, 16, 32, 36). *C. jejuni* is phenotypically and genetically very diverse, and there are many typing techniques available (39), which are useful to a greater or lesser extent for the comparison of strain identity. One problem is that the genome of campylobacters is highly susceptible to genomic instability, which can confound molecular epidemiological investigations over an extended time period, particularly when used to track isolates which have been exposed to environmental stresses, such as those in the poultry farm environment. Techniques like pulsed-field gel electrophoresis (PFGE) are

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particularly susceptible to such effects. Nevertheless, because the time and distances during a flock life are short, genetic instability is not generally an issue. However, such methods are expensive and time-consuming. A second and more serious problem is related to the sampling and efficiency of strain recovery. The fragility of campylobacters under environmental stresses, including temperature (5, 8, 15), atmospheric oxygen (14, 17), and dehydration (10), is well recognized. Experience indicates that even those environmental campylobacters which can be recovered frequently grow poorly and may die during storage. In addition, given the ubiquitous nature of the organism, the multiple potential sources, and the extensive environment of a farm, the processing and typing of sufficiently representative numbers of isolates would be poorly cost-effective. Thus, a novel approach to poultry farm sampling to identify campylobacter sources infecting a flock is required.

The approach we adopted was to develop a molecular probe specific for the strain colonizing chickens in an individual flock. This probe would then be used to screen DNA extracted from environmental samples, collected over the life of the flock and enriched for campylobacters. From the genomic information available, there are several regions of the genome which are sufficiently genetically diverse to provide strain-specific sequences (30). The most well-recognized genetically diverse regions are within the *flaA* gene and have been used as the basis for PCR-restriction fragment length polymorphism (RFLP) (3, 27, 29). In this gene, there are two main regions of sequence variability separated by a conserved sequence. The shorter of the two segments of high variability, the short variable region (SVR), is located between positions 450 and 600 of the *flaA* gene sequence (25).

In this report, we describe the use of the SVR of *flaA* to develop a fluorescently labeled PCR oligonucleotide probe specifically designed to detect the DNA of the strain colonizing a poultry flock in samples taken from the environment. Real-time PCR amplification was used to screen the samples to give a cost-effective approach. Because mismatches between the probe and target affect the kinetics of melting, the melting peaks from this technology are characteristic of a particular probe-target sequence (38), which enables the specificity of the method to be assessed by using strains with known SVR sequences. The sensitivity of the method was also investigated by using bacterial cultures and spiked fecal samples. Finally, proof of principle was tested in a field study of one poultry farm and the results were compared with those obtained with isolates recovered and characterized by PFGE and PCR following conventional culture.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Twenty-four *C. jejuni* strains belonging to 15 different *fla* types (as defined by the method of Ayling and colleagues (3) were included in this study (Table 1). For specificity studies, the following strains were also tested: *C. lari* NCTC 11352; *C. hyointestinalis* NCTC 11608, BR385/04, and BT175/05; *C. upsaliensis* NCTC 12183; *Proteus mirabilis* VLAPr01; *Pseudomonas aeruginosa* VLAPs02; *Escherichia coli* EC341/95; and *Enterococcus faecium* ENT4.

Campylobacter strains were cultured on selective agar (sheep blood agar containing Skirrow's supplement plus actidione and cefoperazone [BASAC]) (36, 37) at 42°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). DNA was extracted from 48-h bacterial growth. *C. jejuni* strains NCTC 11168 and M1 were used for sensitivity tests and for inoculating spiked samples. Other strains were cultured on blood agar at 37°C aerobically for 24 h.

TABLE 1. *Campylobacter* strains selected for SVR and LightCycler analyses

Strain	Source	*fla* RFLP[b] (SmaI PFGE[c]) type
NCTC 11168	Human	1,1 (S33)
81116	Human	2,5 (SR)
Mon1	Human	2,5 (S29)
35/4	Broiler flock	2,5 (SR)
OF1	Broiler flock	1,1 (S92)
118/12	Broiler flock	1,14 (S52)
X69[a]	Broiler flock	1,12 (S115)
X790	Broiler flock	1,12 (S33)
176/2	Broiler flock	7,13 (S56)
35/14	Broiler flock	1,13 (S98)
35/7	Broiler flock	1,13 (S52)
350/3	Broiler flock	1,11 (S72)
98/53	Broiler flock	1,9 (S42)
X937	Broiler flock	3,14 (S33)
172/2	Broiler flock	3,4 (S56)
98/110	Broiler flock	3,7 (S51)
393/14	Broiler flock	3,7 (S132)
306/16	Broiler flock	3,13 (S110)
172/1	Broiler flock	3,13 (S125)
X110	Broiler flock	3,1 (S110)
X133	Broiler flock	6,14 (S111)
X33	Broiler flock	5,2 (S110)
X89[a]	Broiler flock	5,14 (S110)
X65	Broiler flock	6,14 (S111)

[a] Biotyped as *C. coli*; all other strains are *C. jejuni*.
[b] Arbitrary *fla* RFLP type according to Ayling et al. (2).
[c] Arbitrary SmaI PFGE type.

Extraction of DNA. Genomic DNA was extracted from 48-h cultures of individual isolates of *Campylobacter* spp. with Prepman Ultra sample preparation reagent (PE Applied Biosystems, Warrington, United Kingdom) according to the manufacturer's instructions.

Sequence analysis of *flaA* SVR. Amplification of PCR products for sequence analysis was performed with the *flaA*-specific primers recommended by Wassenaar and Newell (39) and Advantage cDNA Polymerase Mix (BD Biosciences, Oxford, United Kingdom). Purification of a PCR product for sequence analysis was undertaken with MicroSpin S-300 HR columns (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturer's instructions.

Design of real-time PCR probes and primers. Two microliters of each bacterial DNA preparation was used as the template for *flaA* SVR PCR with primers FLA242FU and FLA625RU (25) (Table 2). A 35-cycle reaction was used with 1 min of denaturing at 96°C, 1 min of annealing at 52°C, and 1 min of extension at 72°C. The resulting product was approximately 400 bp. Sequence data were generated by using the FLA242FU and FLA625RU primers independently.

The DNA sequences obtained were aligned with that of NCTC 11168 (30) and other available sequences (GenBank) with the Megalign Clustal multiple-alignment program (DNASTAR Inc., Madison, WI). For preliminary studies, on the basis of sequence data, different oligonucleotide probes were designed for two separate *fla* genotypes, as defined by the method of Ayling and colleagues (3). The probe for *fla* type 1,1 (probe M1) (Table 2) matched strain NCTC 11168, while the probe for *fla* type 2,5 (probe M2) matched strains NCTC 81116 and Mon1. Probes were 21 to 24 bases in length and were Cy5 labeled at the 5′ end. Biotin labeling at the 3′ end prevented the probe from acting as a primer during the amplification phase. Primers Fwd and Rv were used to produce *C. jejuni* or *C. coli* SVR-specific amplicons (Table 2). The primers and probes were synthesized by Sigma-Genosys Ltd. (Haverhill, Suffolk, United Kingdom).

LightCycler probe assay. The probe hybridization assay was performed in the LightCycler 2.0 real-time PCR system (Roche Diagnostics Ltd., Lewes, Sussex, United Kingdom). Optimization experiments were performed to determine the most suitable reaction conditions. The *C. jejuni flaA* target sequences were amplified in a 20-μl reaction mixture containing 1× LightCycler-FastStart DNA Master hybridization probe mix (Roche Diagnostics), 4 mM $MgCl_2$, each primer at 0.5 μM, 0.2 μM probe, and 2 μl of lysate. The amplification segment comprised denaturation at 95°C for 10 min, followed by 32 to 35 cycles of 95°C for 10 s, 50°C for 6 s, and 72°C for 6 s with a temperature transition rate of 2°C/s. The melting point (T_m) curve for the annealing of the PCR product with the probe

TABLE 2. Primers and probes used in this study

Primer or probe	Sequence (5′ to 3′)	Position in strain NCTC 11168 *flaA*	Expected annealing temp (°C)	Reference
FLA242FU	CTA TGG ATG AGC AAT TWA AAA T	242–263	55	25
FLA625RU	CAA GWC CTG TTC CWA CTG AAG	643–623		
Primer Fwd	GAT TTG AAA TTC TTG ATT G	447–428	52	This study
Primer Rv	CAA GAA CYA TGC TTC AAG	323–340		
Probe M1	CY5-CAA TAT TGT CAA GTT CTT CCA TTA-BIO[a]	379–356	58	
Probe M2	CY5-CGA TAT TAT CAA GCT CTT CCA TCA-BIO	379–356	62	
Primer Fwd	GAT TTG AAA TTC TTG ATT G	447–428	52	This study
Primer Rv	CAA GAA CYA TGC TTC AAG	323–340		
Probe P1	CY5-CGA TAT TGT CAA GTT CTT CCA TT-BIO	379–357	58	
Primer 1	CTG TTC CAA CTG AAG TTG	638–620	52	This study
Primer 2	GTT TAA CAC GCT TTG AAA CAG	509–529		
Probe P4	CY5-ATT GCA CTT CGC CAC TAG ATG-BIO	562–537	62	

[a] Biotin labeled at the 3′ end to prevent primer extension.

was determined from 40°C to 90°C with a temperature transition rate of 0.1°C/s while fluorescence was monitored continuously in channel F3. Melting peaks were derived by plotting the negative derivative of fluorescence over temperature versus the temperature [−d(F3)/dT versus T]. The highest melting peak temperature should be attained. For DNA quantification, the normalized reporter signal was plotted against the number of cycles. The threshold signal was determined for each amplification plot. Samples whose threshold cycle (C_T) values were >30 were considered negative. The C_T value was plotted against log input DNA to provide standard curves for the quantification of unknown samples.

Sensitivity of detection of bacterial cells. To test the lower detection limit of the real-time PCR amplification and specificity, strains NCTC 11168 and Mon1 were grown overnight and resuspended in phosphate-buffered saline to approximately 10^8 CFU/ml. A 10-fold dilution series was prepared in 2-ml volumes of modified Exeter broth (mEB) (18). DNA was extracted from 1 ml of enriched broth cultures with Prepman Ultra sample preparation reagent. Briefly, a 1-ml aliquot of enrichment broth culture was removed from each dilution and centrifuged at 16,000 × *g* for 10 min to sediment bacterial cells, and the supernatant was discarded. The pellet was resuspended in 200 μl of sample preparation reagent, and the suspensions were heated to 100°C for 10 min on a heating block (Techne Dri Block; Barloworld Scientific Ltd., Stone, United Kingdom). The samples were allowed to cool to room temperature and centrifuged again at 16,000 × *g* for 2 min, and then a 100-μl aliquot of the supernatant was transferred to a fresh tube for analysis. This sample was then used as the template in the LightCycler assay. A negative control comprising a lysate prepared from Exeter broth was used to eliminate false positives. The extracted template (2 μl) was used for the PCR. SVR consensus primers Fwd and Rv, targeting a sequence-variable region of strains NCTC 11168 and M1, were used to amplify the target strain DNA in the diluted samples. Standard curves were constructed by plotting CFU versus the C_T produced for the DNA target. Comparison of PCR amplification efficiencies and detection sensitivities was facilitated by performing a linear regression analysis with LightCycler software (version 3.5). The limit of detection was the lowest dilution reproducibly detected by the PCR. To ensure that the target strain was detectable in a background of genomically unrelated strains, 10^2 to 10^3 CFU of strain NCTC 11168 were mixed with 10^2 to 10^7 CFU of *C. jejuni* strain Mon1.

Preparation of *C. jejuni*-contaminated cecal samples. Ceca (n = 6) were collected from a group of 4-week-old housed commercial broiler chickens known to be negative for *Campylobacter* spp. Ceca (n = 6) were also collected from a second group of birds which had been challenged with *C. jejuni* strain Mon1 (27). The cecal contents were individually placed in 9 ml of mEB. Cultures were enriched at 42°C for 48 h under microaerobic conditions. Colony counts were performed on the enriched samples with BASAC plates. Identification of presumptive campylobacter isolates was based on colony morphology, and identification to the species level by real-time PCR was performed according to the method of Best et al. (4) with the Stratagene MX3005p (Stratagene, Amsterdam, The Netherlands).

Field study. Two adjacently located poultry houses (houses 7 and 8) were sampled on farm A. House 7 was selected as the target house. Each house contained 4,375 birds. An adjoining farm (farm B) with six houses (houses 1 to 6) used the same driveway and office. The two farms were treated as one site with regard to the timing of the flock cycles. Cattle and sheep grazed in a field located adjacent to house 7 of farm A. The farmer on farm A also had a second poultry farm (farm C) located 0.4 km away.

The selected houses on farm A were first visited on the day of filling and then seven more times (at days 21, 23, 27, 30, 34, 36, and 40) during the crop cycle. Feces samples were collected from the poultry houses at each visit, and cecal samples were collected at slaughter. Ceca were also collected from birds on farms B and C at slaughter. Ceca from the previous flock of farm A were also collected at slaughter. At each farm visit, environmental samples were collected from the anteroom, ventilation system, and external structure of the target house and from the concrete apron surrounding the house. In addition, vehicles present on the farm at the time of the visit, fox and mouse droppings on site, cattle and sheep feces in the adjacent field, drainage ditches, a water trough, puddles, the main drive, the adjacent field, and farmers' boots were sampled by a standardized approach (2, 6). Farm environmental samples were added to mEB at the farm in a 1:10 ratio (weight/volume), except for water, where the ratio was 1:3, as recommended by Bull et al. (6). The inoculated broth samples and the fecal samples were immediately transported under chilled conditions to the laboratory, where samples were then immediately incubated for 48 h at 37°C prior to plating on modified CCD agar and incubation at 41.5°C. Pooled fecal and cecal samples were also plated directly onto modified CCD agar. Following enrichment, 1.5-ml aliquots of all enriched samples were transferred to screw-cap Eppendorf tubes and mixed with 200 μl of glycerol prior to storage at −80°C until required.

To prepare samples for DNA extraction, the enriched broth samples were disturbed by gentle shaking and allowed to settle before the transfer of 1 ml of each broth to an Eppendorf tube. The tubes were then centrifuged at 16,060 × *g* for 10 min. Supernatants were discarded, and the pellets were resuspended in 200 μl of Prepman Ultra sample preparation reagent. The samples were heated to 100°C for 10 min, cooled, and then centrifuged before recovering 100 μl of supernatant as previously described. Heavily sedimented samples were resuspended in larger volumes (250 to 400 μl) of sample reagent to facilitate sample suspension.

Isolates recovered from broilers in the target flock in house 7 of farm A (n = 20), the adjacent house (house 8 of farm A) (n = 6), farm C (n = 6), and the previous flock in house 7 of farm A (n = 3) were genotyped by PFGE. Isolates from culture-positive environmental samples (n = 34) were genotyped in the same way. Briefly, chromosomal DNA was prepared from *C. jejuni* isolates according to the method of Gibson et al. (11). DNA was digested overnight with SmaI, and PFGE was performed on a DRIII (Bio-Rad, Hemel Hempstead, United Kingdom) apparatus at 6 V/cm for 23 h with pulse times increasing from 5 to 40 s with standardized parameters as proposed by CAMPYNET (http://www.medvetnet.org/pdf/Reports/PFGE_protocol.pdf). Digital gel images of SmaI digests were analyzed with Bionumerics Software (Applied Maths, Kortrijk, Belgium). Comparison was facilitated by using the Dice coefficient, and position tolerance values were set at 1.6%. Cluster analyses was performed by the unweighted-pair group method using average linkages. Identification of flock types to the species level was performed by real-time PCR as previously described.

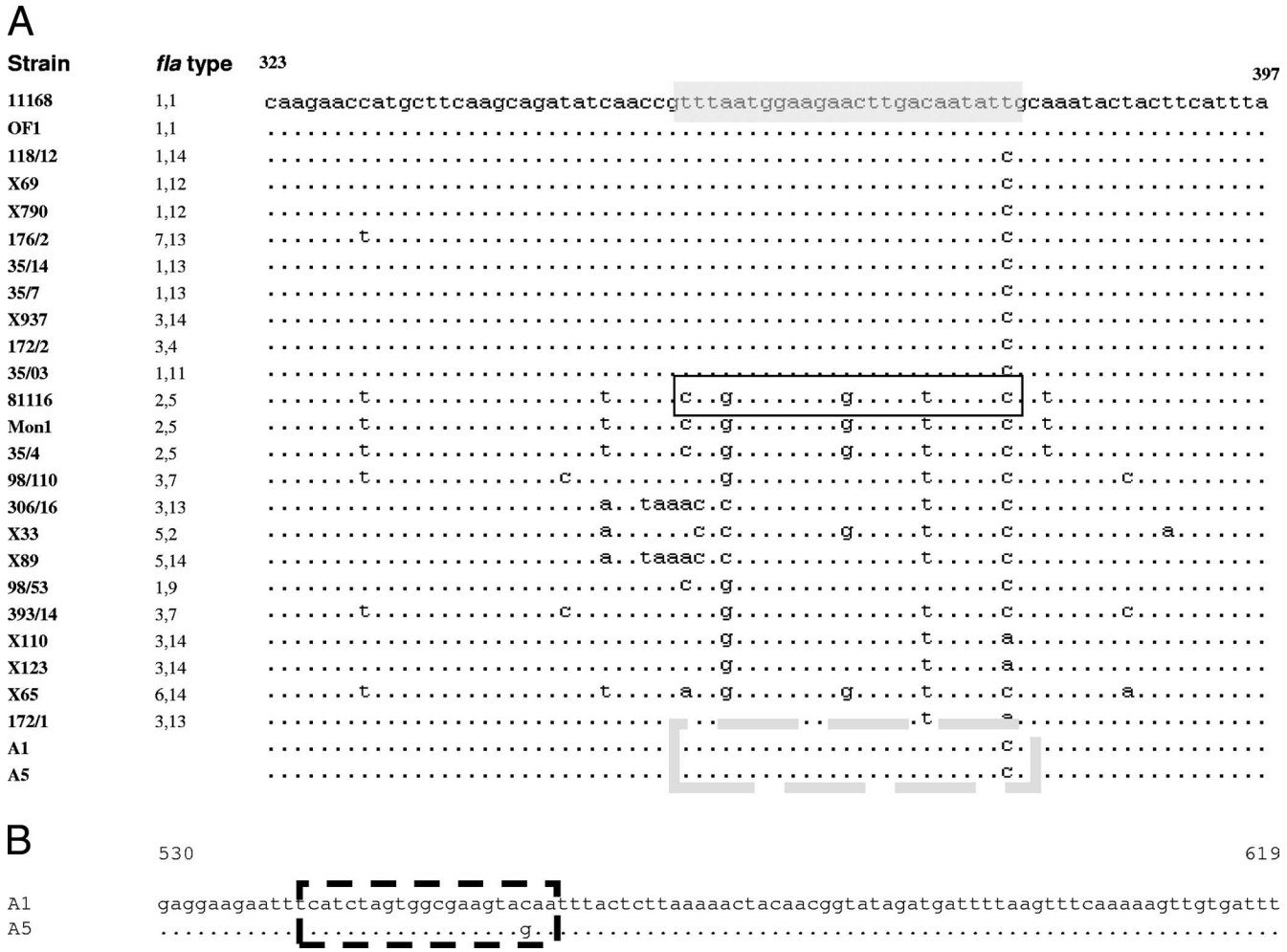

FIG. 1. Nucleotide sequences of the segments of the *flaA* SVRs of strains used for assay development (A) and field study (B). The numbering of nucleotides is as previously described for the genome sequence strain (23), with dots indicating nucleotides identical to those in that sequence. The gray-shaded box contains the sequence targeted by probe M1. The black-boxed sequence is the region targeted by the M2 probe. A1 is the representative strain from the target flock in house 7 of farm A of PFGE type A1. The probe P1 sequence is boxed by gray dashed lines. The sequence shown in panel B is sSVR2 for PFGE types A1 and A5. The probe P4 sequence is boxed by black dashed lines.

Identification of flock-colonizing SVR sequence types. On the basis of PFGE type, eight broiler isolates from flocks in houses 7 and 8 of farm A ($n = 3$), farm C ($n = 1$), the previous crop in house 7 ($n = 2$), and broiler feces in house 7 ($n = 2$) were selected for *flaA* SVR sequence analysis to determine the most suitable probe for differentiating between flock-colonizing types. Although the probe was designed to be specific in sequence for only one SVR type, mismatches with other SVR types could occur. Nevertheless, exact matches to the probe would be expected to produce melting curves which have the highest possible melting temperatures, whereas mismatches would have a lower melting temperature or no melting peak at all. Melting peak profiles were assigned according to the probe name, with the homologous target sequence generally allocated the peak temperature of T1. Nonmatching peaks were then numbered sequentially.

Nucleotide sequence accession numbers. The 24 sequences obtained by PCR in this study have been submitted to GenBank (accession numbers EU104685 to EU104708).

RESULTS

Real-time strain-specific assay development. Initially, a PCR product of approximately 400 bp, corresponding to the *flaA* SVR and flanking sequences, was amplified with FLA242FU and FLA625RU (Table 2) from all 24 *C. jejuni* and *C. coli* strains examined but not from the other *Campylobacter* spp. or other bacterial strains tested (data not shown). The PCR products were sequenced.

A short region of 125 bp within the SVR (sSVR) that was amplified by primers Fwd and Rv (Table 2) was selected for routine sequencing of test strains and used to define the probe sequence. The sSVR was selected for the greatest sequence variability surrounded by conserved sequences for primers, and part of this sSVR is shown in Fig. 1 but without the conserved primer regions.

Considerable nucleotide heterogeneity in the sSVR sequences (Fig. 1) was observed in the strains tested. Comparison of the sSVR sequences indicated some, but not complete, correlation with the *fla* type as defined by PCR-RFLP and indicated that as expected, SVR sequencing has the higher discriminatory power. From this region, probe sequences 21 to 23 nucleotides long were designed to give the greatest discrimination. For example, probes M1 and M2, designed to have the same sequence as strains NCTC 11168 and NCTC 81116, re-

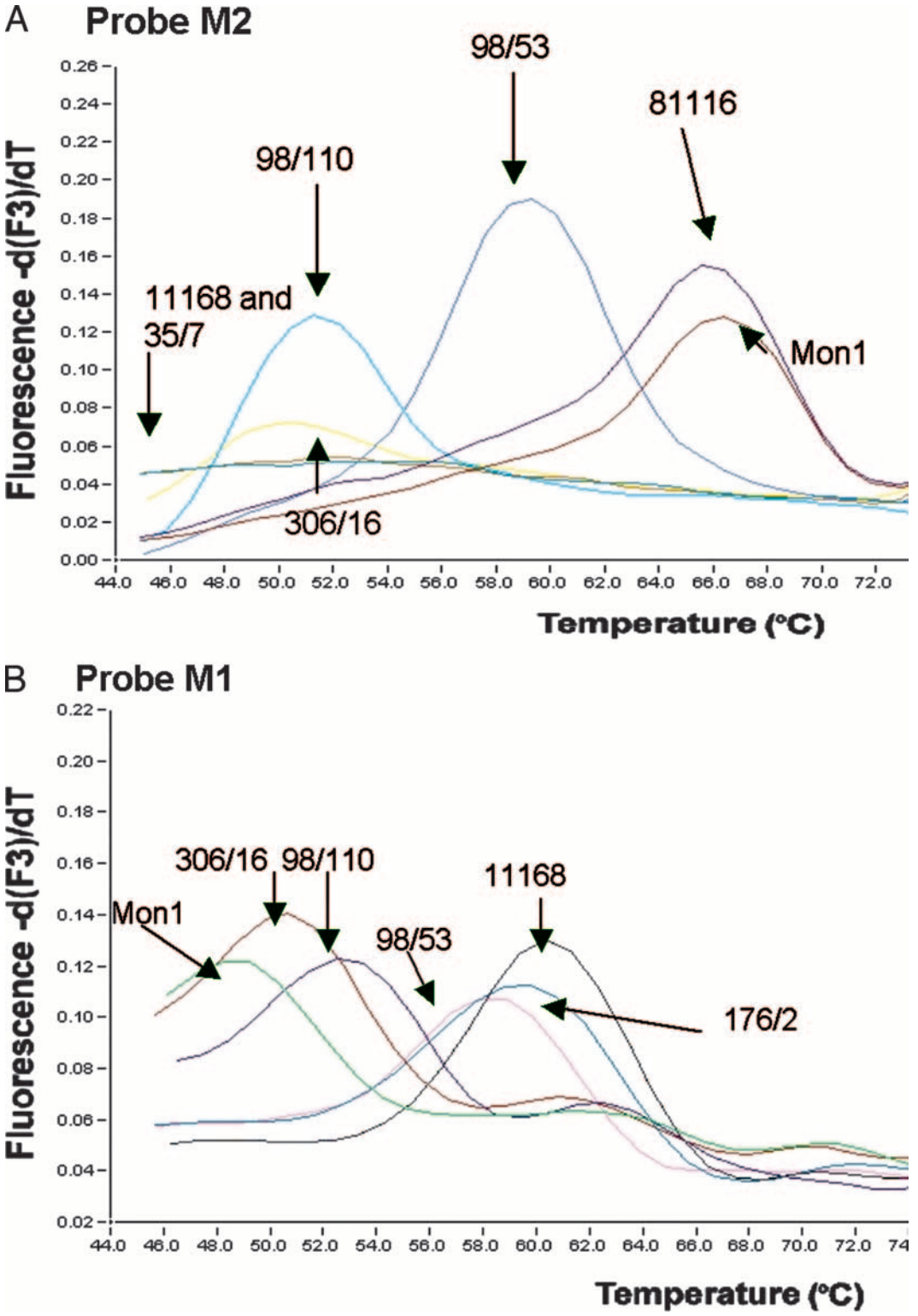

FIG. 2. Representative melting curves produced by probes M2 (A) and M1 (B) with strains with different SVR sequences and *fla* types. The melting curves are distinguishable on the basis of differences in T_m values and indicate sequence differences of one to five nucleotides within the probe region.

spectively, had zero to four nucleotide mismatches with the other *C. jejuni* strains tested (Fig. 1).

In the real-time PCR, these sequence similarities and differences were detected by temperature variations at the apex of the melting curves. For example, PCR products with sequences matching the test probe (i.e., Mon1 and probe M2) formed a stable hybrid and showed a melting curve (Fig. 2A) with a T_m indistinguishable from that of the original strain (in

this example, strain NCTC 81116). In contrast, in the presence of nucleotide differences (i.e., the two nucleotide differences in this region between strains NCTC 81116 and 98/53), a melting curve with a difference in T_m of 7°C was observed (Fig. 2A). The T_m differences observed were dependent on both the number and position(s) of the mutation(s). For probe M1 (Fig. 2B), the T_m obtained with the homologous strain was clearly distinguishable from those obtained with the strains with one (e.g., strain 176/2), three (strains 98/53 and 98/110), and four (strain 306/16) differences. The largest difference in T_m was observed for strains Mon1 (Fig. 2B) and NCTC 81116 (not shown), with a peak at 48.5°C. Both of these strains had five nucleotide differences within the probe-binding region of M1.

Sensitivity and specificity. From the standard curve produced (not shown), the detection limit for *C. jejuni* strain Mon1 in Exeter broth in the LightCycler assay with homologous probe M2 was estimated to be 3.7×10^2 CFU/ml. Similarly, the detection limit for strain NCTC 11168 with homologous probe M1 was 1.3×10^2 CFU/ml. Probe type-specific melting curves were observed for all dilutions, down to and including the limit of detection. No hybridization to the probe was detected in the broth alone. In addition, no PCR product was obtained with the FLA242FU/FLA625RU or Fwd/Rv primer pair and DNA from other *Campylobacter* species (*C. lari*, C *hyointestinalis*, *C. upsaliensis*) or the other bacterial species tested, indicating that the primers were specific for *C. jejuni* or *C. coli*.

The detection limit in chicken fecal samples spiked with strain Mon1 was calculated to be 6.6×10^2 CFU/ml. A single peak, consistent with the M2 probe and the homologous strain, was observed for PCR-positive samples only (not shown). Three of five cecal samples from chickens experimentally infected with 3×10^7 CFU of *C. jejuni* strain Mon1 were identified as positive both by the PCR with primers Fwd and Rv and by real-time PCR with probe M2. In the remaining two cecal samples, no campylobacter-like colonies were recovered and no PCR product or hybridization by real-time PCR was detected with the M2 probe, indicating that these two birds were not colonized.

Application of the probe assay in a field study of a poultry farm. A total of 39 isolates were recovered from the 507 environmental samples collected on and around target farm A. The number of samples from which campylobacters were recovered by culture methods (against the total number taken) for each of the visits was as follows: day 1, 6/88 (6.8%); day 21, 3/55 (5.4%); day 23, 2/85 (2.3%); day 27, 4/75 (5.3%); day 30, 3/39 (7.7%); day 36, 9/60 (15.0%); day 40, 7/53 (13.3%).

Twelve isolates identified as *C. jejuni* were recovered from cecal samples from the target flock (house 7 of farm A) and seven isolates were recovered from the adjacent house (house 8 of farm A) at slaughter. All of these cecal isolates were identical on the basis of SVR sequence analysis. Similarly, all of these isolates were of the same PFGE type (A1) (Fig. 3). The sSVR sequence of this poultry strain was used to design probe P1, which differed by a single nucleotide from the M1 probe (Fig. 1).

Probe P1 was used to screen for the presence of DNA matching the target flock-colonizing type in the fecal and cecal isolates and the stored culture-positive enrichment broth samples from the environmental samples and the corresponding isolates (Table 3).

Probe P1 appeared to detect a variety of strains in these samples, as indicated by variations in the melting curves observed (Fig. 4). In order to analyze these data, a classification scheme was developed for these melting curves. Melting curve type P1.T1 represented the interaction of the probe with the homologous strain from the target flock in house 7. Other melting curves obtained were numbered sequentially.

Probe P1 produced a melting curve of type P1.T1 in all 23 cecal and fecal isolates tested from houses 7 and 8 of farm A (Table 3). The same melting curve was obtained with isolates from the ceca of the flock on farm C. All of these isolates had the same PFGE pattern (A1). However, isolates from the previous flock in house 7, also identified as *C. jejuni*, clearly had a different melting curve (P1.T2) and were reproducibly SmaI resistant by PFGE (A0), indicating that this was not the source of infection in the target house.

Unfortunately, only 36 of 39 isolates were available for investigation because of loss of viability on storage. Probe P1 generated consistent melting curves for both the broth and the isolate in 20 of these 36 matching samples (Table 4). For 19/20 cases, the curve was identical to P1.T1. All of the remaining 13 broth samples gave no detectable PCR product with the Fwd and Rv primers (Table 4), suggesting that campylobacter DNA was not present. Nevertheless, campylobacter isolates were originally recovered from these samples. Interestingly, most of these campylobacter DNA-negative samples were obtained at the beginning of the study. Further investigation suggests that this conundrum is explainable in these early instances by poor conditions of preparation and storage of the broth samples to preserve the DNA. In addition to the culture-positive environmental samples, a further 176 culture-negative samples were tested. The P1.T1 melting curve was detected in three of these samples, suggesting that the probe approach may, in some instances, have enhanced sensitivity over culture.

A comparison of P1 melting curve types against PFGE types for the environmental samples is shown in Table 5. The discriminatory ability of probe P1, calculated by using a numerical index of discrimination (19), was $D = 0.22$, compared with a PFGE value of $D = 0.45$ for the environmental and flock isolates collected. This indicates that the discrimination afforded by PFGE was higher than for probe P1 used alone. Notably, P1 failed to differentiate among the three different PFGE types, A1, A3, and A5. Therefore, sequence analysis of representatives of these three PFGE types was performed. The sequences of the strains representing PFGE types A1 and A3 were homologous to the sequence of the isolate from the flock in target house 7 of farm A. Moreover, closer inspection of the PFGE banding patterns showed six bands in common between PFGE types A1 and A3 (Fig. 3), suggesting genotypic relatedness between these isolates. In contrast, the SVR sequence for the isolate representing PFGE type A5 was different and a second probe, P4 (Fig. 1), was designed by using a second sSVR between nucleotides 497 and 620 of the NCTC 11168 *flaA* gene sequence (Table 2). With all of the eight flock isolates tested with P4, a single melting curve type (P4.T1) was obtained. There was complete congruity between the similarities in the isolates recovered from the flocks as detected by probes P4 and P1 and PFGE (Table 4).

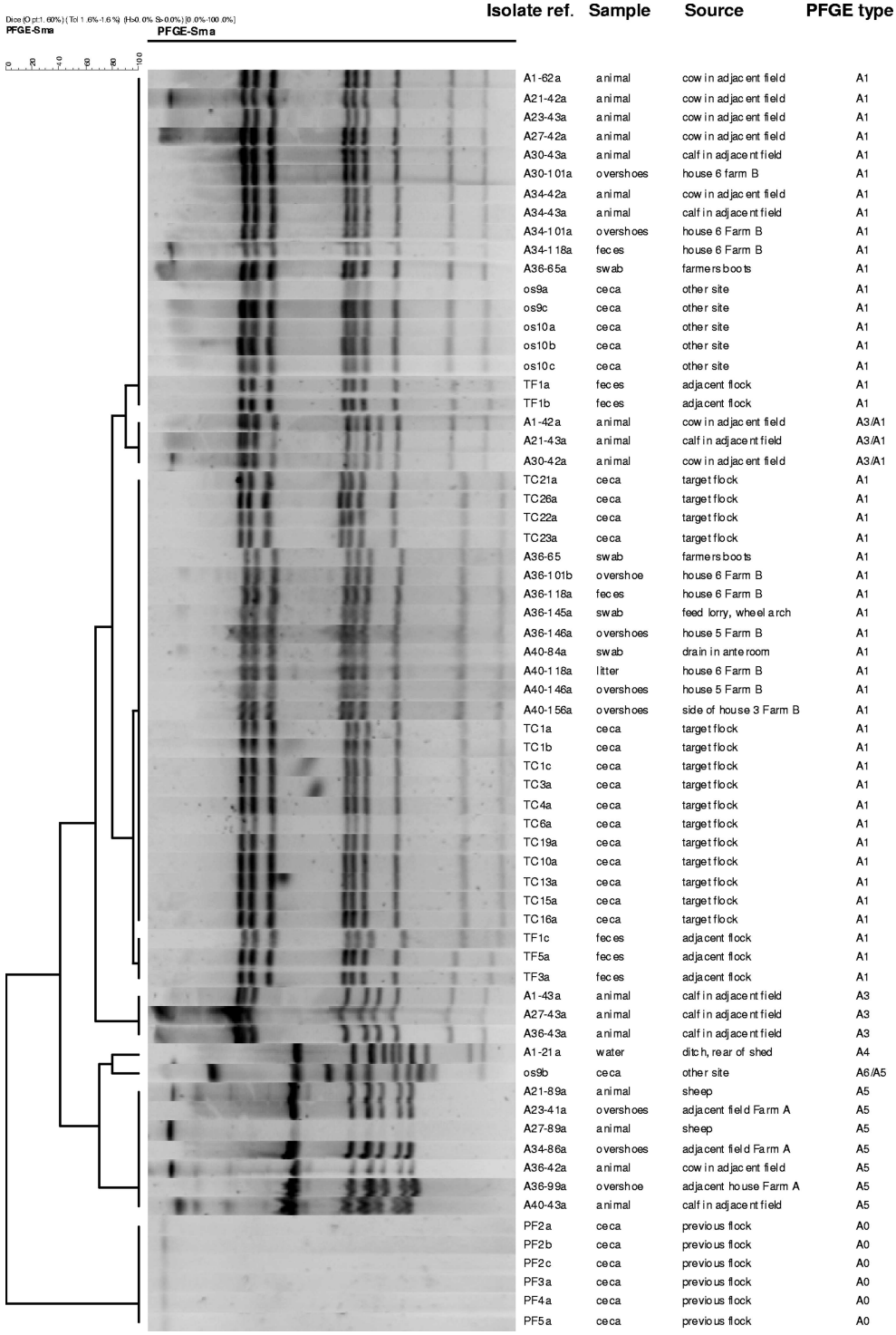

FIG. 3. PFGE of the *Campylobacter* isolates from the flocks and environmental samples associated with farm A. The band position tolerance was set at 1.6%. Strain references (ref.) labeled TC, TF, and PF refer to isolates from target flock ceca, target flock feces, previous flock ceca, and farm C ceca shown in Table 4. Environmental sample numbers (e.g., A1-62a) are prefixed by day of sampling.

Use of this second probe (P4) allowed the differentiation of isolates belonging to PFGE type A5 from A1 and A3, and this enhanced the discriminatory power of the test approach to $D = 0.49$.

Three of 176 environmental samples from which campylobacter could not be recovered by conventional culture methods were positive for the flock-colonizing type DNA with probe P1 (not shown). Screening with probe P4 confirmed a match to the flock-colonizing type for these isolates. A further eight DNAs prepared directly from enriched samples could be am-

TABLE 3. Characterization of poultry cecal and fecal isolates

Sample source	Collection time	Type	Probe (no. of samples tested)	Probe type	PFGE type
Target flock, farm A	Day 40	Feces	P1 (7) P4 (3)	P1.T1 P4.T1	A1
Target flock, farm A	Slaughter	Ceca	P1 (16) P4 (3)	P1.T1 P4.T1	A1
Previous flock, farm A	Slaughter	Ceca	P1 (4) P4 (1)	P1.T2 P4.T2	A0
Flock, farm B	Day 34	Feces	P1 (1) P4(1)	P1.T1 P1.T1	A1
Flock, farm C	Slaughter	Ceca	P1 (6) P4 (1)	P1.T1 P4.T1	A1

plified with the probe-specific primers. However, four of these failed to produce a melting curve while the remainder produced a melting profile (P1.T3/P4.T2) distinct from that of the flock-colonizing type.

Application of the probe approach to track environmental sources of campylobacters for the flock on the poultry farm. Environmental samples producing melting curves identical to P1.T1 and/or P4.T1 were first observed on farm A in calf feces from cattle in an adjacent field at day 1 of sampling. Similar probe-positive samples were observed from this source throughout the sampling period (at days 1, 21, 23, 27, 30, and 34). This positivity was confirmed with isolates with the same melting curve type (P1.T1 and/or P4.T1) and with a PFGE type of either A1 or A3. The genotypic relatedness of these two types, as well as the epidemiological association, strongly suggested that PFGE types A1 and A3 were clonally linked and formed a common pool. Thus, the molecular epidemiological evidence indicated that the strain colonizing the flocks in houses 7 and 8 of farm A was present in the farm environment when the birds were 1 day old and significantly before they became detectably campylobacter positive in at least one of those houses at 36 days of age. Interestingly, there was at least one other strain, distinguishable by PFGE, in the cattle but this was neither detected as DNA nor recovered as isolates from the flocks.

By day 30, the distribution of DNA characterized by the P1.T1 and/or P4.T1 melting curve type in the farm environment was widespread (Table 4; Fig. 4), with probe-positive samples recovered from the house including feces in adjacent farm B, the farmer's boots, and even the wheel wells of a feed truck. This widespread distribution was confirmed by the characterization of isolates from these sources.

As described previously, three enrichment broth samples which were culture negative were also detected by the probe approach. All of these samples were from house 7 of farm A and included a swab from a drain in the anteroom at day 34, the spent litter in the emptied house at day 40, and a swab from tools in the anteroom at day 40. These results are consistent with other culture-positive samples and suggest that, in some instances, the probe approach can detect poorly culturable organisms.

DISCUSSION

The tracking of strains around farms has been a major and successful strategy for targeting interventions against organ-

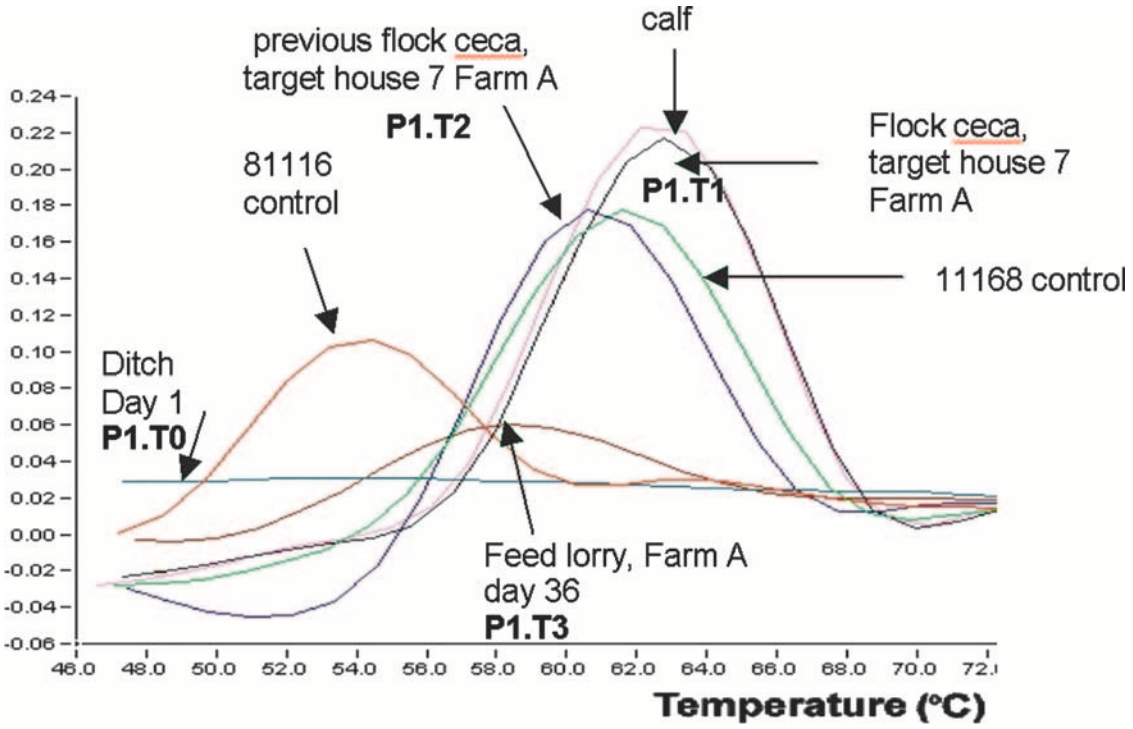

FIG. 4. Representative melting curves produced by probe P1 with farm A flock and environmental samples. Melting curves obtained with control strains NCTC 81116 and NCTC 11168 are also shown.

TABLE 4. Probe and PFGE investigation of environmental sample broths and isolates

Farm sample	Day	Sample type	Probe name	Enrichment broth		Isolate	
				PCR product	Melting curve type	Melting curve type	PFGE type(s)
Ditch, rear of house 7, farm A	1	Water	P1	−		P1.T0	A4
			P4	−		P4.T4	
Field adjacent to farm A	1	Overshoes	P1	−		P1.T1	A5
			P4	−		P4.T2	
Cow in adjacent field, farm A	1	Feces	P1	−		P1.T1	A1, A3
			P4	−		P4.T1	
Calf in adjacent field, farm A	1	Feces	P1	+	P1.T1	P1.T1	A3
			P4	+	P4A.T1	P4.T1	
Cow in adjacent field, farm A	1	Feces	P1	−		P1.T1	A1
			P4	−		P4.T1	
Field adjacent to farm A	1	Overshoes	P1	−			Nonviable
			P4				
Cow in adjacent field, farm A	21	Feces	P1	−		P1.T1	A1
			P4	−		P4.T1	
Calf in adjacent field, farm A	21	Feces	P1	−		P1.T1	A1, A3
			P4	+	P4.T1	P4.T1	
Sheep in adjacent field, farm A	21	Feces	P1	−		P1.T1	A5
			P4	+		P4.T3	
Field adjacent to farm A	23	Overshoes	P1	+	P1.T1	P1.T1	A5
			P4	+	P4.T3	P4.T3	
Cow in adjacent field, farm A	23	Feces	P1	+	P1.T1	P1.T1	A1
			P4	+	P4.T1	P4.T1	
Field adjacent to farm A	27	Overshoes	P1	No broth		P1.T1	A5
			P4	+		P4.T3	
Cow in adjacent field, farm A	27	Feces	P1	+	P1.T1	P1.T1	A1
			P4	+	P4.T1	P4.T1	
Calf in adjacent field, farm A	27	Feces	P1	No broth		P1.T1	A3
Sheep, farm A	27	Feces	P1	No broth		P1.T1	A5
Cow in adjacent field, farm A	30	Feces	P1	−		P1.T1	A1
			P4	−		P4.T1	
Calf in adjacent field, farm A	30	Feces	P1	−		P1.T1	A1
			P4	−		P4.T1	
House 6, farm B	30	Overshoes	P1	+	P1.T1	P1.T1	A1
			P4	+	P4.T1	P4.T1	
Cow in adjacent field, farm A	34	Feces	P1	+	P1.T1	P1.T1	A1
Calf in adjacent field, farm A	34	Feces	P1	+	P1.T1	P1.T1	A1
			P4	+	P4.T1	P4.T1	
Field adjacent to farm A	34	Overshoes	P1	+	P1.T1	P1.T1	A5
			P4	+	P4.T3	P4.T3	
House 6, farm B	34	Overshoes	P1	+	P1.T1	P1.T1	A1
			P4	+	P4.T1	P4.T1	
House 6, farm B	34	Overshoes	P1	+	P1.T1	P1.T1	A1
			P4	+	P4.T1	P4.T1	

Continued on facing page

TABLE 4—*Continued*

Farm sample	Day	Sample type	Probe name	Enrichment broth		Isolate	
				PCR product	Melting curve type	Melting curve type	PFGE type(s)
Cow in adjacent field, farm A	34	Feces	P1	−		P1.T1	A5
			P4	−		P4.T2	
Calf in adjacent field, farm A	36	Feces	P1	−		P1.T1	A1, A3
			P4	−		P4.T1	
Farmer's boots, farm A	36	Swab	P1	−		P1.T1	A1
			P4	−		P4.T1	
Field adjacent to farm A	36	Overshoes	P1	−		Nonviable	
Adjacent house, farm A	36	Overshoes	P1	+	P1.T1	P1.T1	A5
			P4	+	P4.T1	P4.T1	
House 6, farm B	36	Overshoes	P1	+	P1.T1	P1.T1 (1) 2 isolates	A1
			P4	+	P4.T1	P4.T1 (1)	
House 6, farm B	36	Feces	P1	+	P1.T1	P1.T1	A1
			P4	+	P4.T1	ND[a]	
Feed truck wheel wells	36	Swab	P1	+	P1.T3	P1.T1(2)/P1.T3 (2) 3 isolates; 1 mixed	A1
			P4	+	P4.T3	P4.T2 (1)	
House 6, farm B	36	Sludge	P1	+		P1.T1	
			P1	+		P1.T1	A1
Control panel, anteroom of house 7, farm A	40	Swab	P1	−		Nonviable	
Cow in adjacent field, farm A	40	Feces	P1	−		P1.T1	A5
			P4	−		P4.T2	
Calf in adjacent field	40	Feces	P1	+	P1.T1	P1.T1	A1, A5
			P4	+	P4.T1	P4.T2	
Drain, anteroom, farm A	40	Swab	P1	+	P1.T1	P1.T1	A1
			P4	ND		P4.T1	
House 6, farm B	40	Overshoes	P1	+	P1.T1	P1.T1	A1
			P4	ND		P4.T1	
House 5, farm B	40	Overshoes	P1	+	P1.T1	P1.T1	A1
			P4	+		P4.T1	
House 3, farm B	40	Overshoes	P1	+	P1.T1	P1.T1	
			P4	ND		P4.T1	A1

[a] ND, not done.

isms like *Salmonella* in poultry flocks. However, for campylobacters, such a strategy is limited by the ubiquitous nature of the organism in this environment and its poor recoverability from such sources. An alternative and novel approach has therefore been developed. This approach exploits the genetic diversity of these organisms and aims to use real-time PCR technology to identify DNA specific for the flock-colonizing strain in environmental samples.

The approach adopted was to develop a LightCycler assay based on oligonucleotide probes targeting the SVR of the *flaA* gene of the *Campylobacter* strain colonizing the target poultry flock. Two striking advantages of real-time PCR are its rapidity and the low cost of analysis. The specificity of such assays is dependent on the similarity between the melting curves of the probe and homologous DNA compared with various test samples. Real-time PCR assays for the detection of *Campylobacter* spp. (24) or *C. jejuni* (34) in poultry fecal samples and carcass rinses (7, 23, 31, 35) have recently been reported. However, the real-time PCR described in this report aims to detect DNA specific to a target campylobacter strain.

The SVR of the *flaA* gene was selected as the target for identifying the target strain. The genetic variability of this DNA region is well recognized, has previously been adopted for strain-typing purposes (25), and has recently been used to improve the discriminatory power of multilocus sequence typing (9). One possible issue with such a method is the propensity

TABLE 5. Comparison of melting curves and PFGE genotypes of environmental samples

Probe and melting curve type	No. of samples with SmaI PFGE type:						Total
	A1	A3	A4	A5	Mixed A1-A3	Mixed A5-A1	
P1.T1	20	2		9	2	1	34
P1.T2							
P1.T3							
P1.T0			1				1
Mixed P1.T3	1						1
P4A.T1	18	2		1	2		23
P4A.T2	1			3		1	5
P4A.T3				4			4
P4A.T4			1				1

for genetic instability in this region. However, given the geographical and temporal limitations involved, genetic instability should be minimized.

The specificity of the assay was investigated both in silico against known sequences from 10 strains and then in the laboratory with those strains. The probes could detect one to five nucleotide differences on the basis of the melting curves observed. This variation was also determined by the locations, as well as the number, of the polymorphisms. The sensitivity of the method was determined with samples spiked with homologous strains to be 1×10^2 to 4×10^2 CFU/ml with two different probes (or 1 to 12 genome equivalents per reaction) for bacterial cultures (40). Estimates of 5×10^2 to 1×10^5 campylobacters/ml (g) in food and environmental samples (20, 23, 24) have been previously reported with similar assays, so this level of test sensitivity appears appropriate.

The purpose of this assay development was to identify potential reservoirs and sources of strains colonizing the poultry flock in the farm environment. The major advantage of a real-time PCR approach is that samples or isolates can be tested in large numbers rapidly and cheaply compared with other typing-based methods. The approach was therefore tested on a single farm to provide proof of principle. The strategy adopted was to sample a target flock, its farm environs, and any associated flocks throughout the flock's life. The poultry farm selected (farm A) was complex, with a directly adjacent poultry farm (farm B) having shared access and an associated poultry farm (farm C). In addition, there were other domestic animal species in adjacent fields. Ceca from all three flocks were sampled at slaughter, as well as feces from flocks on farms A and B during the growing period. The environmental sampling included six visits to the farm and the collection of 507 samples, which were then enriched and cultured, and any isolates and the enrichment broth samples were stored frozen in the presence of a cryopreservative. Of these environmental samples, only 39 yielded campylobacter isolates and three of these isolates were unrecoverable from storage, supporting previous experience indicating that environmental campylobacter isolates are highly stressed and poorly culturable.

Sixteen isolates were recovered from the target flock at slaughter. All were of the same PFGE type, supporting previous evidence that in intensively reared broiler flocks in the United Kingdom colonization is generally restricted to one or two strains (3). The SVR of a representative flock strain was sequenced, and an sSVR probe (probe P1) was designed. This probe identified identical strains colonizing flocks on farm B at least 6 days earlier and on farm C at or about the time of slaughter but not in the previous flock on farm A, indicating that colonization may have been initiated on farm B and then transmitted to the adjacent poultry houses on farm A and that the previous flock on farm A was not a source. The specificity of the probe in these cases was confirmed by PFGE on the various isolates.

Subsequently, all of the culture-positive environmental enrichment broth samples were tested with probe P1 by the real-time PCR method. Surprisingly, particularly in the earlier samples collected, no *Campylobacter* DNA was detected in a number of these broth samples, despite evidence of a positive culture. However, investigation suggested that the handling and storage of the broth samples for future DNA testing were not optimal, which could have explained this anomaly. In particular, it is considered vital that aliquots of enrichment broth samples be made and frozen as soon as possible following completion of incubation. For later visits to the farm, the recovery of probe-positive samples was substantially improved and at least three probe-positive but culture-negative samples were identified, suggesting that, in some instances, the sensitivity of the probe approach was greater than that of the culture method.

A variety of melting curves were obtained, indicating the presence of at least four strains (Fig. 4) detectable by probe P1 and that the melting curve characteristic of the target flock strain (P1.T1) could be detected in many of the environmental samples. PFGE of the available isolates confirmed this observation but indicated that the same melting curve was detectable in isolates with a clearly different PFGE pattern (A5), suggesting incomplete specificity of this probe. This was confirmed to be a consequence of similarity in the SVR sequences of these two strains. Subsequently, a second probe, P4, with significantly improved discriminatory power was designed on the basis of another part of the SVR of the A5 strain.

With the combined discriminatory power of the two probes, the strain, which eventually colonized the target flock, could be tracked around the farm environment. Clearly, this strain was persistently carried by cattle in an adjacent field. It has previously been established that cattle are frequently colonized by *C. jejuni* and *C. coli* (28) and that the species and serotypes observed in cattle strains are similar to those that can affect humans. A link between cattle colonization and flock colonization has also been identified (6, 13), although the direction of transfer could not be established and the presence of other animals on the farm is considered a high-risk factor for campylobacter-positive flocks (1).

This investigation has also provided evidence that campylobacter contamination of the farmer's boots provided the route of transmission from the cattle in the adjacent field to the poultry farm environment, probably causing colonization in houses on adjacent farm B first and then leading to colonization of the flock on farm A. Campylobacters recovered from farmers' boots were identified in a recent study as a likely source of flock infection (22), and a report from the Food Standards Agency entitled *Evidence for the Effectiveness of Bio-*

security to Exclude Campylobacter from Poultry Flocks (available at http://www.food.gov.uk/safereating/microbiology/flocks/), as well as a systematic review of the literature (1), clearly indicates that farmers are the major vehicle for tracking campylobacters into poultry houses. There is also a possible role for the feed truck in transmission as a P1.T1-positive signal was recovered from a wheel well, but there were many other strains also present and a clear relationship to the poultry-colonizing strain could not be confirmed.

In conclusion, a real-time PCR assay using the LightCycler platform for the identification of specific *C. jejuni* SVR types in DNA-containing lysates from enrichment broth samples has been developed. This method has been used to investigate the on-farm sources of campylobacters colonizing a target poultry flock. By retrospectively testing environmental samples which had been collected, enriched, and stored frozen throughout the flock-growing period, the approach could overcome the problems associated with poor recoverability of environmental campylobacter strains and the major resource costs associated with strain typing. In the farm investigated, cattle in a field adjacent to the target poultry houses were carrying a strain identical to that which later colonized the flock. The evidence from this preliminary study was supported by the characterization of isolates from the flock and the farm environment. A larger trial of multiple farms has recently been completed, and preliminary data confirm the proof of principle of the probe approach. These results may allow the environmental sources of campylobacters in poultry flocks to be prioritized and to enable targeted interventions to be developed.

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